Kinases and miRNAs in the pathogenesis of small B cell lymphomas

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Chapter 7

miRNA profiling of B-cell subsets: specific miRNA profile for germinal center B-cells with variation between centroblast and centrocytes

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Abstract

MicroRNAs (miRNAs) are an important class of small RNAs that regulate gene expression at the post-transcriptional level. It has become evident that miRNAs are involved in hematopoiesis and hematopoietic malignancies. The aim of our study was to establish miRNA profiles of naïve, germinal center (GC) and memory B-cells sorted from tonsils and validate their expression patterns in lymphoid tissues. Quantitative (q)RT-PCR profiling revealed that several miRNAs were elevated in GC B-cells, including miR-17-5p, miR-106a and miR-181b. miR-150 was more than 10-fold lower in germinal center B-cell as compared to the other two subsets. MiRNA in situ hybridization (*ISH*) on tonsil tissue sections confirmed findings from the profiling work. Interestingly, gradual decrease of miR-17-5p, miR-106a, and miR-181b staining intensity from the dark to the light zone was observed in GC. Also, within the GC a minority of cells showed a much stronger cytoplasmic staining of miR-150 in part of the blasts in the dark zone. Induction of miR-150 lead to reduced c-Myb, Survivin and Foxp1 expression levels in the Burkitt lymphoma cell line, DG75. In conclusion, miRNA profiles of naïve, GC and memory B-cells were established and validated by miRNA *ISH*. Within the GC cells a marked difference was observed between the light and the dark zone.

Introduction

MiRNAs are a new class of small RNAs, of 19-23 nucleotides that were discovered less than two decades ago [1;2]. When incorporated into Argonaute (Ago) proteins to form the RNA induced silencing complex (RISC), these small RNAs can negatively regulate genes at the post-transcriptional level by either triggering translational repression or direct cleavage of mRNAs [3]. MiRNA expression can be highly tissue specific [4] and miRNAs are known to regulate many cellular processes including proliferation, apoptosis, cell cycle, differentiation and hematopoiesis [5-8].

The distinct and dynamic expression pattern of miRNAs during hematopoiesis reflects the importance of this class of small RNAs in determining the final state of differentiation for hematopoietic cells [9-12]. Alteration in expression level of merely one miRNA in hematopoietic stem cells can skew the differentiation process towards a specific hematopoietic cell type [13]. For this reason, it is anticipated that aberrant expression of miRNAs may give rise to hematopoietic malignancies. MiRNAs that are important in hematopoietic development and those that may contribute to malignancies have gradually been characterized in the past few years.

C-Myb, a transcription factor known to play a key role in B-cell maturation, has been recently shown to be a direct target of miR-150 [14]. Ectopic expression of miR-150 in hematopoietic stem cells lead to blockage of the transition of pre-B to pro-B stage [15]. The PU.1 transcription factor, essential in hematopoietic lineage development, and activation-induced cytidine deaminase (AID), a protein that is important in class switch recombination of the immunoglobulin locus, have been demonstrated as targets of miR-155 [16;17]. As a consequence of miR-155 down regulation, inefficient germinal center response causing production of less IgG_1 cells was observed. Li *et al.* demonstrated that miR-181a can fine tune T-cell sensitivity during the maturation process by directly targeting SHP-2, PTPN22, DUSP5, and DUSP6, four components of the T-cell receptor signaling pathway [18]. In parallel with the discoveries of important hematopoietic miRNAs, it has become evident that miRNAs are involved in the pathogenesis of leukemias and lymphomas. For example, miR-21, miR-92, miR-150, miR-155 and miR-222 are reported to be deregulated in chronic lymphocytic leukemia (CLL) [19;20]. Overexpression of miR-155 as well as the miR-17-92 cluster has been documented for several B-cell lymphomas, including diffuse large B-cell lymphoma (DLBCL) and Hodgkin lymphoma (HL) [21-23]. In a transgenic mouse model, ectopic expression of miR-155 leads to lymphoblastic leukemia/high grade lymphoma [24].

Recently, several studies have been carried out to establish miRNA profiles of hematopoietic cells [25], but a detailed validation of the miRNA profile throughout the

germinal center response has not been published. Thus, the aim of our study was to characterize the miRNA expression profile of normal B-cell subsets, which includes naive B-cells, germinal center (GC) B-cells and memory B-cells. As there were only limited differences at the gene expression level reported between centroblast and centrocyte using microarray analysis [26;27], both cell types were grouped as GC B-cells in our study. By comparing these profiles, GC B-cell specific miRNA expression patterns were defined. Findings from the profiling work were validated with miRNA *in situ* hybridization (*ISH*) technique. GC B-cell associated miRNA expression patterns were established and these miRNAs were analyzed in B-cell lymphomas by miRNA *ISH*.

Materials and methods

FACS sorted normal B-cell subsets

Normal B-cell subsets were sorted by FACS from 3 different tonsil samples, essentially as described previously [28]. Briefly, mononuclear cells were isolated by Ficoll-Isopaque density gradient centrifugation. The collected cell suspension was stained with FITC-conjugated anti-human IgD, PE-conjugated anti-human CD19 (both from Dako, Glostrup, Denmark), and allophycocyanin-conjugated anti-human CD38 (BD Pharmingen, NJ, USA). Using a FACS aria (BD Biosciences, San Jose, USA) for sorting, naive B-cells ($CD19^+$, IgD^+, $CD38^-$), GC B-cells ($CD19^+$, IgD^-, $CD38^+$) and memory B-cells ($CD19^+$, IgD^-,$CD38^-$) were isolated, gated for single cells by forward and sideward scattering. After sorting, the B cell subsets were lysed in RNA-Bee (Tel-Test Inc. Frindswood, Texas, USA) and stored at -80ºC, until further processing.

Tissue sections

Paraffin-embedded tissue samples of 4 tonsils and 4 progressive transformed germinal center (PTGC) were obtained from patients diagnosed in the Department of Pathology and Laboratory Medicine, University Medicine Center Groningen. All protocols for obtaining and studying human tissues and cells were approved by the institution's review board for human subject research and diagnosis was in accordance with WHO classification guidelines [29].

qRT-PCR and clustering analysis

RNA was isolated from normal B-cell subsets with Nucleospin RNA 11 (Macherey-Nagel, Düren, Germany) as described previously [28]. qRT-PCR profiling for 183 mature miRNAs (Applied Biosystems, Foster City, USA) were carried out according to manufacturers' protocols. Unsupervised clustering analysis was performed using *Genesis* [30]. A heatmap was generated by mean normalization of experiments and

genes, followed by average linkage clustering for all miRNAs that showed a Ct value of <35 in at least one of the three B-cell subsets. Mean and median Ct values were similar in all three B-cell subsets and mean Ct values were used for normalization of miRNA expression ($\Delta Ct = Ct_{miRNA} - Ct_{mean}$). Relative expression levels were determined with the formula $2^{-\Delta Ct}$. Selection of GC B-cell specific miRNAs was performed by the following criteria: 1) miRNAs with $2^{-\Delta Ct}$ greater than 2 in at least one of the subsets, i.e. miRNAs with a relatively high expression level and 2) miRNA showing at least 5 fold difference among the subsets. For miR-150 monoplex qRT-PCR (Applied Biosystems, Foster City, USA), U6 was used for normalization.

miRNA *in situ* hybridization (*ISH*)

MiRNA *ISH* was performed as reported previously [31]. Briefly, 10 digoxigenin (DIG) labeled locked nucleic acid (LNA) probes antisense to miR-15b, miR-17-5p, miR-21, miR-25, miR-29a, miR-93, miR-106a, miR-146a, miR-150 and miR-181b (Exiqon, Vedbaek, Denmark) were used for overnight hybridization on normal tissue sections at 55°C. Detection was accomplished with anti-DIG alkaline phosphate Fab fragment followed by nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) color development (Roche, Switzerland).

Immunohistochemistry (IHC)

The slides were deparaffinized and endogenous peroxidase was blocked by incubation with 3% H_2O_2 for 10 minutes. Antigen retrieval was performed according to various protocols of the manufacturers. Immunostaining was performed using antibodies against Survivin (Cell Signaling Technology, Boston, MA), Foxp1 and c-Myb (Abcam, Cambridge, UK) at a dilution of 1:50-1:400. Signals were amplified by incubation with the appropriate Horseradish Peroxidase-conjugated antibodies for 60 minutes and the reactivity was visualized by diaminobenzidin.

Cell culture, transfection and western blotting

Burkitt lymphoma (BL) cell line, DG75 [32], were cultured in RPMI 1640 supplemented with ultraglutamine, 100 U/ml penicillin/streptomycin, and 10% fetal calf serum (Cambrex Biosciences, Walkersville, USA). Transfection of DG75 with synthetic miR-150 mature duplex (Ambion, Austin, USA) was performed using the A-23 program of the Amaxa nucleofector I device (Amaxa, Gaithersburg, USA) with nucleofection solution V. 24h post transfection, cells were lysed, separated in 10% SDS-polyacrylamide gel and immunoblotted for c-Myb, Foxp1 and Survivin with the same antibodies as used for IHC.

Results

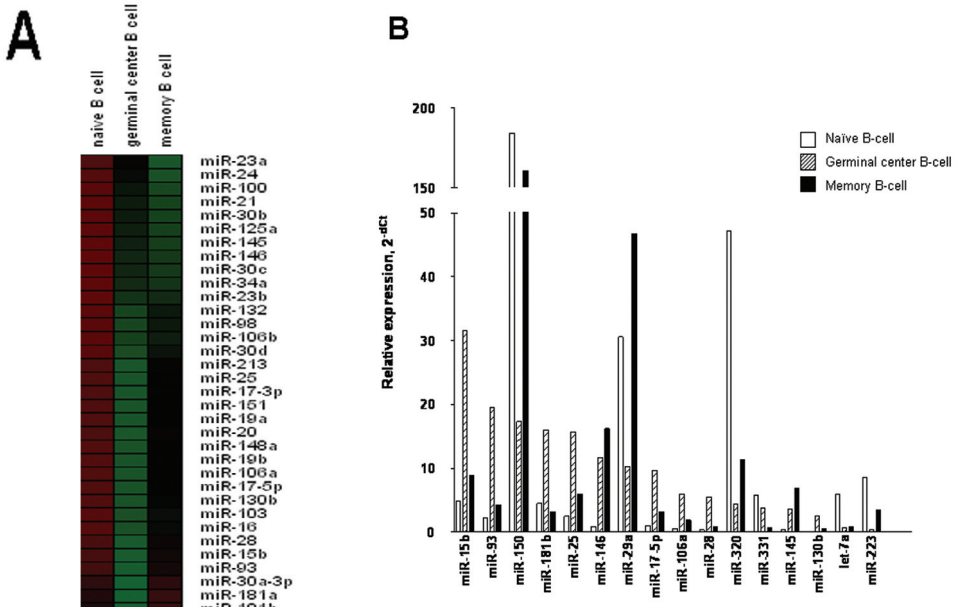


Figure 1. miRNA profiling of naïve, germinal center and memory B cells. A) Heatmap of 86 miRNAs which were expressed (Ct<35) in at least one of the normal B cell subsets and showed differential expression among the subsets. **B)** 16 miRNAs showed at least 5 fold difference when compared to germinal center B cells. The first 9 miRNAs and miR-21 (control, not shown in graph) were selected for *ISH*.

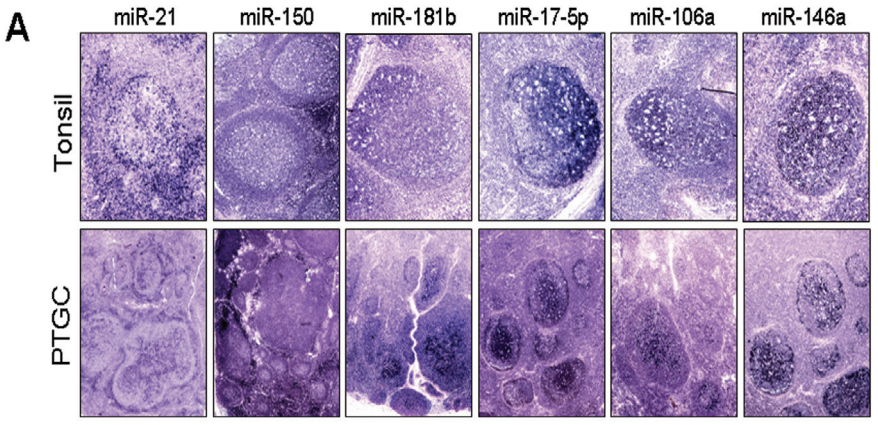
miRNA profiling for normal B-cell subsets from tonsils

Out of 183 miRNAs assessed, 86 miRNAs were expressed (Ct<35) in at least one of the normal B-cell subsets. A heatmap of these 86 miRNAs is given in Figure 1A. To identify miRNAs that were important throughout the germinal center response, we focused on the miRNAs which showed at least 5 fold differences between GC and non GC B-cells, resulting in 16 miRNAs (Figure1B). Eight of these 16 miRNAs, namely miR-15b, miR-93, miR-181b, miR-25, miR-17-5p, miR-106a, miR-28 and miR-130b showed increased expression levels in GC B-cells while 4 miRNAs, miR-150, miR-29a, miR-320 and miR-223 showed a lower expression level in GC B-cells. Expression levels of miR-145 and miR-146 increased gradually during the GC transit whereas the expression levels of miR-331 and let-7a decreased during GC transit (Figure 1B).

miRNA *in situ* hybridization on tonsils

In view of the differential miRNA expression among the normal B-cell subsets and our main interest in GC B-cells, 9 miRNAs which were most abundant and differentially expressed in GC B-cells (first 9 miRNA listed from the left, Figure 1B) were chosen for miRNA *ISH*. As miR-21 did not differ much among the 3 B-cell subsets (data not shown), this miRNA was chosen as a control. Staining for 4 miRNAs (miR-15b, miR-93, miR-25 and miR-29a) failed in all cases for unknown reasons. This failure was not related to the abundance of the miRNAs in GC B-cells as determined by qRT-PCR profiling.

In general, miR-21 worked well as a control as there was no variation in staining intensities of GC structures and the mantle zone in tonsil tissue sections. According to morphology and distribution pattern in the tonsil, cells which showed strongest miR-21 staining were likely to be interfollicular T-cells (Figure 2A). MiRNA *ISH* results showed weak cytoplasmic staining of miR-150 in GC as compared to the mantle zone, while miR-17-5p, miR-106a, and miR-181b stained stronger in GC structures. This suggests a higher expression of miR-17-5p, miR-106a and miR-181b and lower expression of miR-150 in GC B-cells as compared to naïve and memory B-cells located in the mantle zone (Figure 2A). These results were consistent with our miRNA profiling results. For the staining of miR-146a our miRNA *ISH* depicted a stronger staining of miR-146a in the germinal center (Figure 2A), mainly contributed by staining in all of the centroblasts in the dark zone of the GC, as compared to a weaker signal in the mantle zone (Figure 2B). Since naive B-cells, which represent a major population in the mantle zone had much lower levels of miR-146a as compared to the other 2 subsets (Figure 1B), this might explain the apparently stronger staining in the GC B-cell dark zone (Figure 2A). Interestingly, variation in staining intensities was observed within the GC structures for several miRNAs. Gradual decrease of miR-17-5p, miR-106a, and miR-181b staining was observed from the dark to the light zone in the GC (Figure 2A). miR-150 demonstrated



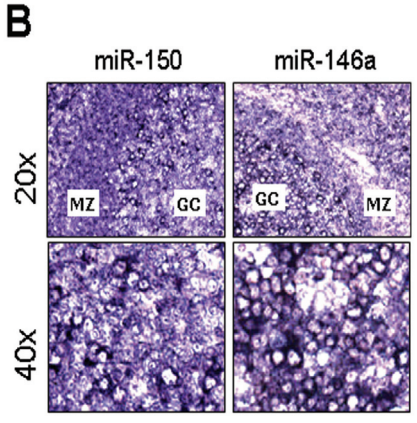


Figure 2. miRNA expression patterns in normal tissue samples. A) miRNA *ISH* in tonsils and PTGCs, 10x magnification for all tonsils, 2x and 4x magnification for the first 3 and latter 3 PTGCs, respectively. **B)** Close up pictures for miRNA staining patterns seen in GC, MZ and centroblast of normal tonsils. *ISH*, *in situ* hybridization. PTGC, progressive transform germinal center. GC, germinal center. MZ, mantle zone.

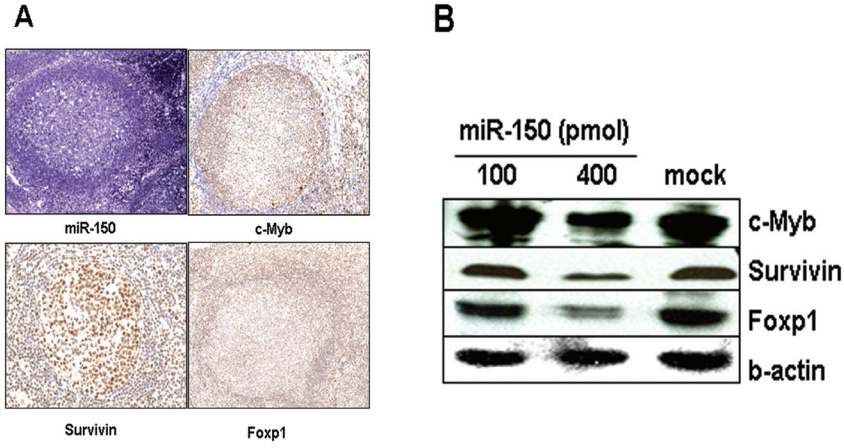


Figure 3. miR-150 expression levels and its possible role in B-cell growth/survival. A) Low miR-150 expression levels in B cell lymphoma cell lines. **B)** Immunohistochemistry staining showing that the expression of c-Myb and Survivin but not Foxp1 are inversely correlated to miR-150 in tonsils, all 10x magnification except 20x magnification for Survivin. **C)** Down regulation of c-Myb, Survivin and Foxp1 upon miR-150 over expression in DG75 cell line.

the strongest staining in part of the blasts only in the dark zone, whereas the light zone, containing the centrocytes, stained even weaker than the naive and memory B-cells in the mantle zone (Figure 2B). In the four PTGC cases, staining patterns were similar to those observed in tonsils. Cells with highest intensity of miR-21 staining were also likely to be T-cells while blast cells showed the strongest staining for miR-17-5p, miR-106a, miR-146a, miR-181b and remarkably also for miR-150 (Figure 2A).

miR-150 expression levels and its possible role in B-cell homeostasis

In view of the dynamic expression patterns of miR-150 throughout the germinal center reaction and its absence in proliferation centers of CLL cases [20], we investigated the possible role of miR-150 in regulation of B-cell homeostasis. One proven target for miR-150, c-Myb [14], and two predicted miR-150 targets, Survivin and Foxp1, were examined by immunohistochemical staining. The c-Myb and Survivin staining patterns were inversely correlated with miR-150 ISH staining patterns in tonsils (Figure 3A), supporting the predicted targeting by miR-150. On the other hand, the staining pattern of Foxp1 did not show a clear inverse pattern with miR-150 ISH (Figure 3A). To confirm targeting by miR-150 we transfected the BL cell line, DG75, which has a GC B-cell origin and low miR-150 expression level. By transfection with synthetic oligonucleotides (mature miR-150 duplex), expression of miR-150 was induced in DG75 (confirmed by qRT-PCR, data not shown). Western blot analysis of cell lysates showed repression of c-Myb, Survivin and Foxp1 in the cells transfected with miR-150 (Figure 3B).

Discussion

Results of miRNA *ISH* on tonsil sections confirmed differential expression of several miRNAs as identified by the miRNA profiling work. More than 5 fold changes in expression levels of miR-17-5p, miR-106a, miR-146a, miR-150 and miR-181b were found in germinal center B-cells as compared to naïve and memory B-cells. A GC B-cell specific expression pattern was confirmed by miRNA *ISH* staining. To our knowledge, this is the first report on distinct miRNA expression patterns in tonsils demonstrating *ISH* as a powerful tool to study cell or compartment specific miRNA expression.

MiRNA *ISH* revealed a distinct staining pattern in the GC structures with decreasing miR17-5p, miR-106a, and miR-181b levels from the dark zone to the light zone. This difference was most likely due to the differences in expression levels between centroblasts and centrocytes. In previously reported gene expression profiling studies to distinguish expression profiles of B-cells during germinal center transit, Klein *et al.* found thousands of differences among naïve, germinal center and memory B-cells, but only 19 differentially expressed genes between CD77- and CD77+ GC B-cells [27]. CD77 is generally accepted as a marker to discriminate between centroblast and centrocytes [33;34]. Consistent with these findings, Hogerkorp and Borrebaeck also found

no differences in gene expression profiles between centroblasts and centrocytes [26]. Lack of differences in these studies raised the question if CD77 can be used as a reliable marker to discriminate between centroblasts and centrocytes. In view of the dynamics of GC B-cells to shift from the dark to the light zone [35] and due to current limitations in isolating true centroblasts and centrocytes, miRNA *ISH* on normal tissue represents a powerful tool to study putative differences in miRNA expression levels in these two closely related B-cell populations.

MiR-17-5p and miR-106a are both members of the same seed family, namely the miR-17 family which also includes miR-17-5p, miR-20a, miR-20b, miR-93, miR-106a and miR-106b. These miRNAs share the same seed sequences and they most likely target the same genes and hence exert similar effects. It has been shown that miRNAs from this miR-17 seed family negatively regulate expression of cyclin dependent kinase inhibitor p21/CDKN1A, allowing cells to overcome the G1 cell cycle checkpoint [36]. As high expression levels of miR17-5p and miR-106a were observed specifically in centroblasts in the dark zone of GC while p21 is 30 fold down regulated in centroblast, we suggest that these miRNAs are essential for centroblasts to progress from G1 to S phase of the cell cycle, by down regulating p21/CDKN1A [26;27].

The expression level of miR-146a increases dramatically when naive B-cells enter the germinal center and maintains high when exit from the germinal center. It has been reported that proinflammatory cytokines and microbial components can induce expression of miR-146a in the acute monocytic leukemia cell line, THP-1. Luciferase reporter assays pointed that miR-146a is a nuclear factor-κB (NF-κB) dependent gene and by targeting IL-1 receptor associated kinase (IRAK1) and TNF receptor-associated factor 6 (TRAF6), miR-146a causes a negative feedback for the Toll-like receptor (TLR) signalling pathway [37]. As NF-κB plays a key role in B-cell homeostasis, not only implicated in immature B cell but also in maintaining GC function [38], our miR-146a strong staining pattern in GC correlates with the role of NF-κB in GC. However, the exact function of miR-146a in this pathway and how it might affect B-cells survival should be further investigated.

MiR-181b contains the same seed sequence with miR-181a. MiR-181a has been proven to play a role in hematopoietic differentiation, in favor of B-cells [13] and its expression was regulated during T-cell maturation [18]. We observed dynamic expression of miR-181b also during the germinal center transit, where its expression level peaks during the germinal center reaction and falls back to the level similar to that of naive B-cells upon exit from the germinal center. For miR-181a, qRT-PCR profiling revealed a 2 fold enrichment in GC B-cells. This indicates that besides miR-181a, miR-181b, a member of the same miRNA seed family might be crucial in B-cell differentiation as well.

Our group has previously demonstrated that miR-150 is highly expressed in the majority of CLL tumor cells, but not in the proliferation centers (46). CLL cells are considered to be of memory B-cell origin [39]. The high expression in CLL is consistent with the high miR-150 expression levels we found in memory B-cells sorted from normal tonsil and the lack in proliferation centers is consistent with the low levels in GC structures of tonsil. Recently, a key role has been established for miR-150 in B-cell development [14;15]. Overexpression of miR-150 blocks B-cell maturation by inhibiting the transition of cells from pro-B to pre-B stage and also induces a slight but significant increase in apoptotic rate [15]. Consistent with this finding, Xiao *et al.* demonstrated that ectopic miR-150 expression in pro-B cells resulted in an increase in cell death [14]. It was concluded that miR-150 exerts this effect by suppression of its target, c-Myb [14], a transcription factor which plays an important role during B-cell development, maintenance of proliferation as well as cell cycle control of hematopoietic cells [40-43]. C-Myb has already been proven to be important for transition of pro-B to pre-B stage and also for maintenance of follicular B-cells, but interestingly, not for mantle zone B-cells [41]. This coincides with our findings, where in the mantle zone, expression of c-Myb indeed was weak while expression of miR-150 that negatively regulates c-Myb was found to be relatively higher. Similarly, in tonsil sections, Survivin showed an inverse staining pattern with miR-150. For Foxp1, however, such an inverse staining pattern was not obvious from our stainings on normal tissue, because expression of Foxp1 was also seen in the mantle zone. Nevertheless, it is still possible that Foxp1 is regulated by miR-150 since Foxp1 knockout mice shared a comparable pro-B to pre-B transition blockade phenotype with mice ectopically expressing miR-150 in hematopoietic cells [15;44]. According to our Western blotting results, induction of miR-150 resulted in downregulation of c-Myb, Foxp1, and Survivin, supporting the targeting by miR-150. Nonetheless, we cannot exclude the possibility of an indirect regulation pathway for downregulation of Survivin and Foxp1.

In conclusion, we have determined a miRNA profile of naïve, germinal center and memory B-cells sorted from normal tonsils. MiRNAs that were differentially expressed have been identified and verified, for the first time, by miRNA *ISH* on tonsil tissue sections. Remarkably, miRNA *ISH* revealed variation in expression levels within the GC B-cells and warrants a further elucidation of the miRNA profile of centroblasts and centrocytes. Based on the dynamic expression throughout the germinal center transit, the inverse relationship with proliferation, as well as its targeting of c-Myb, Survivin, and Foxp1, we propose that miR-150 plays a pivotal role in cell homeostasis of B-cells and lymphomas.

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